Cell-specific proteomic analysis in *Caenorhabditis elegans*

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Proteomic analysis of rare cells in heterogeneous environments presents difficult challenges. Systematic methods are needed to enrich, identify, and quantify proteins expressed in specific cells in complex biological systems including multicellular plants and animals. Here, we have engineered a *Caenorhabditis elegans* phenylalanyl-tRNA synthetase capable of tagging proteins with the reactive noncanonical amino acid *p*-azido-L-phenylalanine. We achieved spatiotemporal selectivity in the labeling of *C. elegans* proteins by controlling expression of the mutant synthetase using cell-selective (body wall muscles, intestinal epithelial cells, neurons, and pharyngeal muscle) or state-selective (heat-shock) promoters in several transgenic lines. Tagged proteins are distinguished from the rest of the protein pool through bioorthogonal conjugation of the azide side chain to probes that permit visualization and isolation of labeled proteins. By coupling our methodology with stable-isotope labeling of amino acids in cell culture (SILAC), we successfully profiled proteins expressed in pharyngeal muscle cells, and in the process, identified proteins not previously known to be expressed in these cells. Our results show that tagging proteins with spatiotemporal selectivity can be achieved in *C. elegans* and illustrate a convenient and effective approach for unbiased discovery of proteins expressed in targeted subsets of cells.

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In a complex eukaryote like *Caenorhabditis elegans*, cell heterogeneity restricts the usefulness of large-scale, mass spectrometry-based proteomic analysis. Enriching for specific cells is challenging, and researchers cannot systematically identify low-abundance proteins expressed in specific cells from whole-organism lysates. Cell-selective bioorthogonal noncanonical amino acid tagging (cell-selective BONCAT) offers a way to overcome these limitations (1, 2). We have previously engineered a family of mutant *Escherichia coli* methionyl-tRNA synthetases (MetRSs) capable of appending the azide-bearing L-methionine (Met) analog L-azidonorleucine (Anl) to its cognate tRNA in competition with Met (3, 4). Because Anl is a poor substrate for any of the natural aminoacyl-tRNA synthetases, it is excluded from proteins made in wild-type cells but is incorporated readily into proteins made in cells that express an appropriately engineered MetRS. Controlling expression of mutant MetRSs by expression only in specific cells restricts Anl labeling to proteins produced in those cells. Tagged proteins can be distinguished from the rest of the protein pool through bioorthogonal conjugation of the azide side chain to alkynyl or cyclooctynyl probes that permit facile detection, isolation, and visualization of labeled proteins. This strategy has been used to selectively enrich microbial proteins from mixtures of bacterial and mammalian cells. For example, Ngo et al. (5) found that proteins made in an *E. coli* strain outfitted with a mutant MetRS could be labeled with Anl in coculture with murine alveolar macrophages, which were not labeled. Using similar approaches, Grammel et al. (6) identified virulence factors from *Salmonella typhimurium* that were expressed in the course of infection of murine macrophages, and Mahdavi et al. (7) profiled *Yersinia enterocolitica* proteins that were injected into HeLa cells. In a complementary approach, Chin and coworkers (8) recently reengineered orthogonal *Methanosarcina barkeri* and *Methanosarcina mazei* pyrrolysyl-tRNA synthetase/tRNA pairs for codon-selective incorporation of a cyclopropene lysine derivative into proteins made in *E. coli*, *Drosophila melanogaster* ovaries, and HEK293 cells; however, this technique requires the expression of both exogenous aminoacyl-tRNA synthetases and tRNAs. Here, we configure cell-selective BONCAT for cell-specific proteomic analysis in the nematode *C. elegans* (Fig. 1*A*). We first demonstrate that restricted expression of a mutant *C. elegans* phenylalanyl-tRNA synthetase (*Ce*PheRS) can label proteins with *p*-azido-L-phenylalanine (Azf; Fig. 1*B*) with spatiotemporal selectivity in the live worm. We then show that cell-selective BONCAT combined with stable-isotope labeling of amino acids in cell culture (SILAC) provides a convenient and effective approach for unbiased discovery of proteins uniquely expressed in a subset of cells.

Significance

The emergence of mass spectrometry-based proteomics has revolutionized the study of proteins and their abundances, functions, interactions, and modifications. However, it is difficult to monitor dynamic changes in protein synthesis in a specific cell type within its native environment. Here we describe a method that enables the metabolic labeling, purification, and analysis of proteins in specific cell types and during defined periods in live animals. Using *Caenorhabditis elegans*, we show that labeling can be restricted to body wall muscles, intestinal epithelial cells, neurons, pharyngeal muscle, and cells that respond to heat shock. By coupling our methodology with isotopic labeling, we successfully identify proteins—including proteins with previously unknown expression patterns—expressed in targeted subsets of cells.

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Data deposition: The vectors generated in this study have been deposited in the Addgene database, www.addgene.org (Addgene nos. 62598 and 62599).

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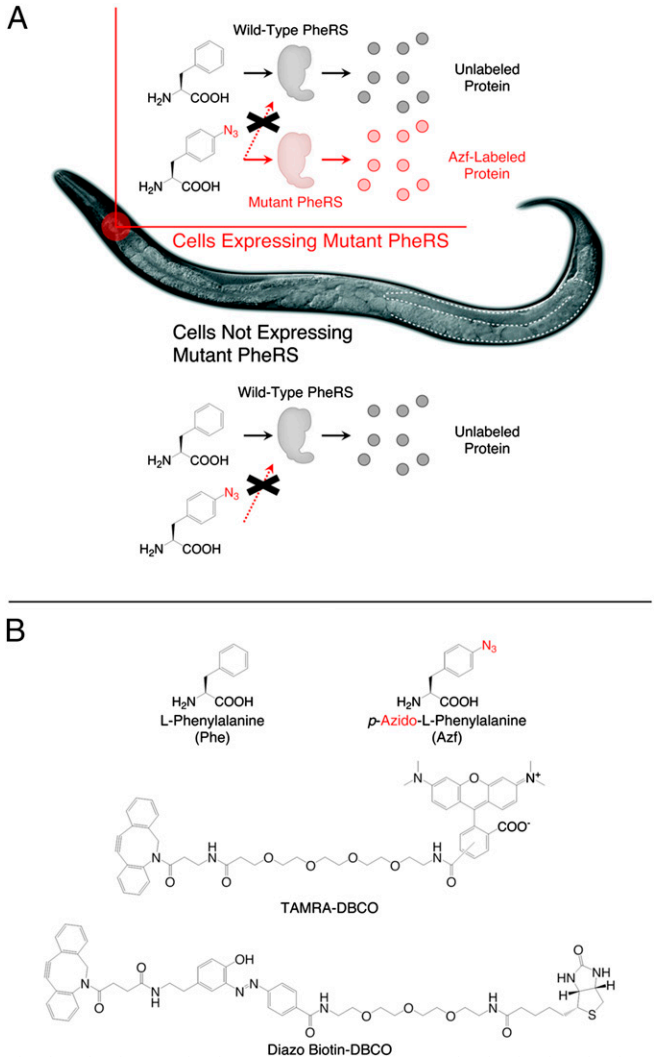

Fig. 1. Cell-selective BONCAT analysis in *C. elegans*. (*A*) A mutant *C. elegans* PheRS is capable of tagging proteins with the reactive noncanonical amino acid Azf. Spatiotemporal selectivity is achieved by controlling expression of the mutant synthetase using cell-selective promoters in transgenic lines. Proteins synthesized in cells that do not express the synthetase are neither labeled nor detected. (*B*) Structures of amino acids and probes used in this study: L-phenylalanine (Phe), *p*-azido-L-phenylalanine (Azf), dibenzocyclooctyne-functionalized tetramethylrhodamine (TAMRA-DBCO), and sodium dithionite-cleavable dibenzocyclooctyne-functionalized biotin (Diazo Biotin-DBCO).

Results and Discussion

Engineering a *C. elegans* PheRS Capable of Activating Azf. We focused our attention on the heterotetrameric *Ce*PheRS because we could not prepare healthy transgenic *C. elegans* strains that express mutant *E. coli* MetRSs. Furthermore, we found that *C. elegans* variants of the mutant *E. coli* MetRSs that we had used to activate Anl in our previous experiments showed no activity toward Anl (*SI Appendix*, Table S1). *Ce*PheRS catalyzes esterification of L-phenylalanine (Phe; Fig. 1*B*) to its cognate tRNA (CetRNA^{Phe}) to form phenylalanyl-tRNA. A conserved "gatekeeper" threonine (Thr412 [the first methionine in the alpha subunit of *Ce*PheRS, isoform A, exon 3 is designated as residue 1 ("Met1")], *C. elegans* numbering; Fig. 2*A* and *SI Appendix*, Fig. S1) in the alpha subunit has been proposed to play a key role in determining substrate specificity in both prokaryotic and eukaryotic PheRSs (9, 10). Therefore, we hypothesized that mutating this residue to smaller residues should enable *Ce*PheRS to activate and charge the larger azide-bearing Phe analog Azf to CetRNA^{Phe}. To screen for such an enzyme, we cultured KY14 [pKPY93/pKPY1XX], a phenylalanine-auxotrophic strain of *E. coli* that expresses mutant forms of *Ce*PheRS, in M9 minimal medium supplemented with different concentrations of Phe and Azf (Fig. 2*B*). To assess *Ce*PheRS activity toward Azf, we detected Azf-labeled proteins by conjugation to dibenzocyclooctyne-functionalized tetramethylrhodamine (TAMRA-DBCO; Fig. 1*B*) and subsequent SDS/PAGE–in-gel fluorescence scanning. Although

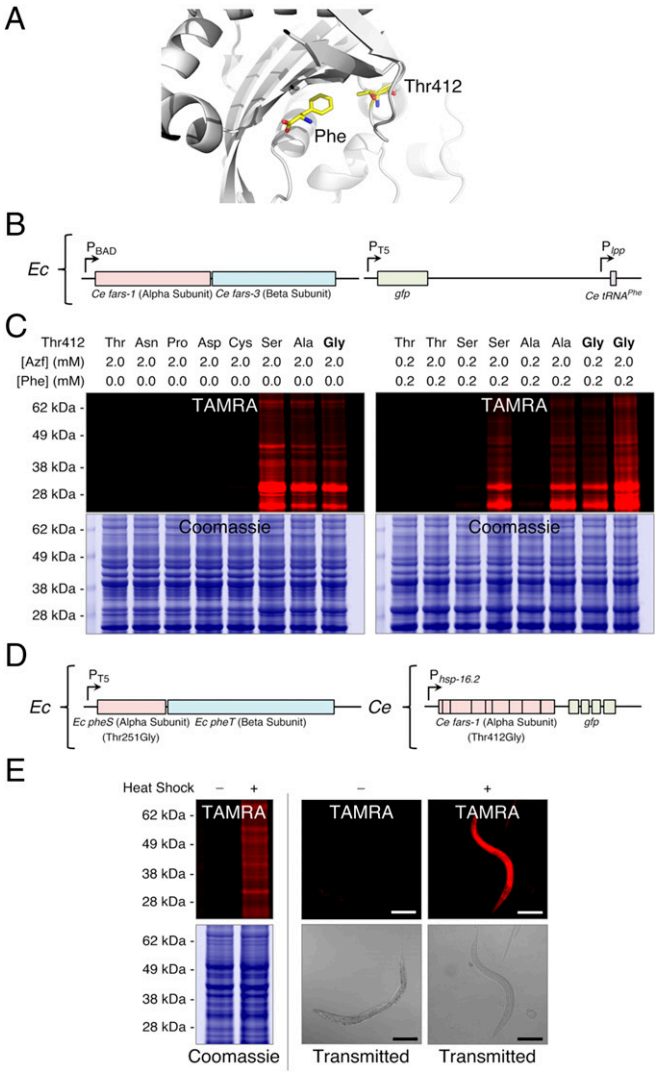

Fig. 2. (*A*) Active site of eukaryotic PheRS with bound Phe (based on PDB ID code 3L4G). (*B*) To screen for a mutant *Ce*PheRS that activates Azf, we used KY14[pKPY93/pKPY1XX], a phenylalanine-auxotrophic strain of *E. coli*. This strain houses two compatible plasmids: (*i*) pKPY93 encodes CetRNA^{Phe} under constitutive *E. coli* murein lipoprotein (*lpp*) promoter control and IPTG-inducible (P_{T5}) 6xHis-tagged GFP and (*ii*) pKPY1XX encodes both *Ce*PheRS alpha and beta subunits under arabinose-inducible (P_{BAD}) control. (*C*) SDS/PAGE and in-gel fluorescence scanning detection of strain-promoted conjugation of TAMRA-DBCO to Azf-labeled proteins derived from KY14[pKPY93/pKPY1XX] lysates. (*D*) We fed KY14[pKPY514] to *hsp-16.2*::Thr412Gly-*Ce*PheRS animals. This *E. coli* strain harbors pKPY514, a plasmid encoding Thr251Gly-*Ec*PheRS under IPTG-inducible (P_{T5}) control. *hsp-16.2*::Thr412Gly-*Ce*PheRS *C. elegans* express both the mutant alpha subunit and GFP under control of the *hsp-16.2* promoter. (*E*) In-gel fluorescence scanning of TAMRA-DBCO–treated lysates (*Left*) and fluorescence microscopy of TAMRA-DBCO–treated fixed animals (*Right*) showed that only heat-shocked worms exhibited ubiquitous Azf labeling 24 h after heat shock. (Scale bars: 100 μm.)

several mutants (Thr412Ser, Thr412Ala, Thr412Gly) showed evidence of labeling with Azf, only the Thr412Gly mutant (Thr412Gly-*Ce*PheRS) displayed robust labeling in cells treated with equimolar amounts of Phe and Azf (Fig. 2*C*). We confirmed by in vitro ATP-PPi exchange assays that Thr412Gly-*Ce*PheRS is highly selective toward Azf: it activates Azf more than 20-fold faster than its canonical substrate Phe (*SI Appendix*, Table S2). Thus, Azf labeling does not require depletion of Phe from an animal's diet, making cell-selective labeling feasible in live worms. Although Thr412Gly-*Ce*PheRS also activates tryptophan threefold faster than Phe, MALDI-TOF mass spectrometry measurements of tryptic GFP peptides did not detect misincorporation of tryptophan or any other canonical amino acid when GFP was expressed in media supplemented with either Phe or Azf (*SI Appendix*, Fig. S2). Collectively, these results suggest that Thr412Gly-*Ce*PheRS selectively activates Azf with catalytic efficiency similar to that observed in the activation of Phe by wild-type *Ce*PheRS. Introducing the Thr412Gly mutation into PheRSs of other eukaryotic cells including human also permits Azf activation (*SI Appendix*, Fig. S3). From these observations, we conclude that Thr412Gly-*Ce*PheRS is the best aminoacyl-tRNA synthetase candidate for cell-selective labeling in *C. elegans*. Although we generated transgenic *C. elegans* by DNA injection into the syncytial germ line in this work, inducible or cell-selective genome editing technologies could be used to quickly and efficiently generate transgenic animals because a single mutation in *Ce*PheRS is sufficient for Azf activity (11).

Characterizing Azf Labeling in *C. elegans*. To evaluate the performance of Thr412Gly-*Ce*PheRS in *C. elegans*, we first generated transgenic *C. elegans* lines that express both the mutant alpha subunit and GFP under control of the *hsp-16.2* promoter (Fig. 2*D*). *hsp-16.2* encodes a 16-kDa protein that is induced in multiple tissues in response to heat shock and other stresses (12). Upon heat shock, we expected that the mutant alpha subunit would be expressed and form a hybrid heterotetramer with the endogenous beta subunit to produce fully active Thr412Gly-*Ce*PheRS. In our initial feeding experiments, we did not detect strong Azf labeling in transgenic animals when Azf was added exogenously to either liquid culture or solid agar plates. However, we found that these lines could be labeled by replacing their normal food source (e.g., *E. coli* OP50) with bacteria whose proteins contain the noncanonical amino acid of choice in a fashion analogous to isotopic labeling (13–15). We first labeled bacteria by culturing KY14[pKPY514], a phenylalanine-auxotrophic strain of *E. coli* that expresses the *E. coli* variant of Thr412Gly-*Ce*PheRS (Thr251Gly-*Ec*PheRS), in M9 minimal medium supplemented with Azf (*SI Appendix*, Fig. S4). The extent of replacement of Phe by Azf in total *E. coli* protein was determined by amino acid analysis to be ∼50% (*SI Appendix*, Fig. S5). We next fed labeled bacteria to *hsp-16.2*::Thr412Gly-*Ce*PheRS worms previously grown on OP50. We induced heat shock in worms grown at 20 °C by 1-h exposure to 33 °C and cleared external as well as ingested bacteria by washing worms with S medium over a period of 30 min. We could not detect *E. coli* protein (even the overexpressed Thr251Gly-*Ec*PheRS) in processed *C. elegans* lysate (*SI Appendix*, Fig. S6). Both in-gel fluorescence scanning of TAMRA–DBCO-treated lysates and fluorescence microscopy of TAMRA–DBCO-treated fixed animals revealed that only heat-shocked worms exhibited ubiquitous Azf labeling 24 h after heat shock (Fig. 2*E*). We detected labeled proteins as early as 1 h after heat shock (shorter times were not tested). Moreover, we observed no differences in behavior, development, or survival in worms fed with Azf-labeled *E. coli* versus worms fed with unlabeled *E. coli* for up to 72 h after heat shock (longer times were not tested).

Labeling Spatially Defined Protein Subpopulations. The core concept of cell-selective BONCAT is that restricting expression of mutant aminoacyl-tRNA synthetases by using promoters active only in specific cells restricts noncanonical amino acid labeling to those cells. Enrichment of labeled proteins permits examination of proteomic changes in those cells. Encouraged by the performance of *hsp-16.2*::Thr412Gly-*Ce*PheRS worms, we next tested cell-specific expression of Thr412Gly-*Ce*PheRS by generating transgenic *C. elegans* lines that express both the mutant alpha subunit and GFP under control of promoters shown previously to be active specifically in the 95 body wall muscle cells (*myo-3*; ref. 16), the 20 intestinal cells (*ges-1*; ref. 17), neurons (*rab-3*; ref. 18), and the 20 pharyngeal muscle cells (*myo-2*; ref. 19) (Fig. 3*A*). We first fed Azf-labeled bacteria to each of these animals. We also observed no differences in behavior, development, or survival in worms fed with Azf-labeled *E. coli* versus worms fed with unlabeled *E. coli* for up to 72 h (longer times were not tested). We then treated fixed worms with TAMRA-DBCO to visualize sites of Azf incorporation. Fluorescence microscopy revealed that labeling in *myo-3*::Thr412Gly-*Ce*PheRS, *ges-1*::Thr412Gly-*Ce*PheRS, *rab-3*::Thr412Gly-*Ce*PheRS, and *myo-2*::Thr412Gly-*Ce*PheRS worms was confined to the body wall muscle, intestine, neurons, and pharyngeal muscle, respectively (Fig. 3*B* and *SI Appendix*, Figs. S7 and S8).

Identifying Pharyngeal Muscle-Specific Proteins. We next investigated whether proteins isolated from worms with cell-specific Thr412Gly-*Ce*PheRS fit characteristics of the targeted cell type. We were particularly interested in the *C. elegans* pharynx, a widely used model to study organ formation during embryogenesis (20). The pharynx is a tube-like muscular pump that concentrates, grinds, and transports bacteria from the mouth to the intestine and comprises 68 cells: 9 epithelial, 4 gland, 9 marginal, 20 muscle, 20 neuronal, and 6 valve cells. We aimed to identify proteins expressed in pharyngeal muscle cells of *myo-2*::Thr412Gly-*Ce*PheRS worms by using a combined cell-selective BONCAT and SILAC approach. We first triply labeled food by culturing KY33[pKPY514], an arginine-, lysine-, and phenylalanine-auxotrophic strain of *E. coli*, in M9 minimal medium supplemented with "heavy" arginine ($^{13}C_6{}^{14}N_4{}^{1}H_{14}{}^{16}O_2$), heavy lysine ($^{13}C_6{}^{15}N_2{}^{1}H_{14}{}^{16}O_2$), and Azf. We next fed these bacteria to fourth larval stage *myo-2*::Thr412Gly-*Ce*PheRS animals previously grown on "light" OP50. According to our model of the cell-selective BONCAT method, all newly synthesized proteins in the animal should contain both heavy arginine and heavy lysine, but only newly synthesized proteins made in pharyngeal muscle cells—the cells that express Thr412Gly-*Ce*PheRS—should contain Azf. This model gives rise to four classes of proteins: (*i*) light preexisting proteins synthesized in pharyngeal muscle cells before the shift in food source, (*ii*) light preexisting proteins synthesized in nontargeted cell types, (*iii*) heavy newly synthesized proteins—labeled with Azf—made in pharyngeal muscle cells after the shift in food source, and (*iv*) heavy newly synthesized proteins made in nontargeted cell types. The use of heavy labels enables relative quantitation of newly synthesized (heavy, H) and preexisting (light, L) peptides. Because enrichment concentrates heavy peptides derived from Azf-labeled proteins, cell selectivity can be quantified by comparing H/L ratios for "enriched" and "unenriched" proteins derived from the same worm sample. Proteins with the highest values of $(H/L)_E/(H/L)_U$ should have originated from pharyngeal muscle cells. Following 24 h of labeling through feeding, worm lysates were incubated with sodium dithionite-cleavable dibenzocyclooctyne-functionalized biotin (Diazo Biotin-DBCO; Fig. 1*B*), and Azf-containing proteins were isolated by streptavidin affinity chromatography (Fig. 3*C* and *SI Appendix*, Figs. S9–S11). Enriched and unenriched samples were resolved by SDS/PAGE and subjected to

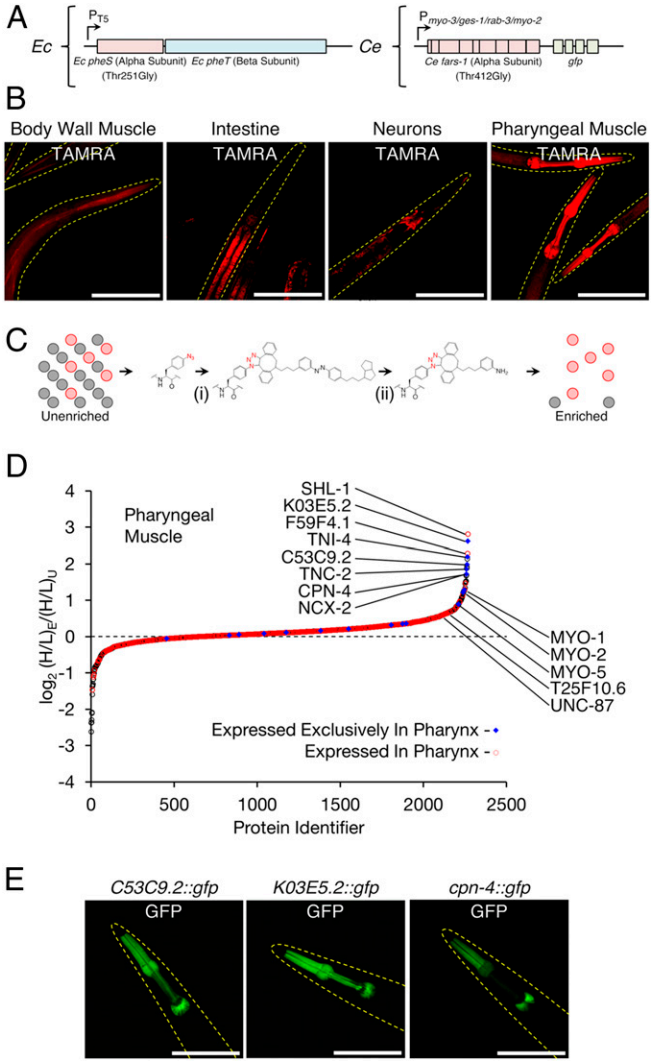

Fig. 3. (*A*) We fed *E. coli* strain KY14[pKPY514] to *myo-3*::Thr412Gly-CePheRS, *ges-1*::Thr412Gly-CePheRS, *rab-3*::Thr412Gly-CePheRS, and *myo-2*::Thr412Gly-CePheRS animals. These transgenic *C. elegans* strains express both the mutant PheRS alpha subunit and GFP under control of the *myo-3*, *ges-1*, *rab-3*, or *myo-2* promoters, respectively. (*B*) Fluorescence microscopy revealed that labeling in *myo-3*::Thr412Gly-CePheRS (*Left*), *ges-1*::Thr412Gly-CePheRS (*Center Left*), *rab-3*::Thr412Gly-CePheRS (*Center Right*) and *myo-2*::Thr412Gly-CePheRS (*Right*) worms was localized to body wall muscle, intestine, neurons, and pharyngeal muscle, respectively; the surrounding tissues were not labeled. (*C*) Tagged proteins were distinguished from other proteins through conjugation of the azide side chain to probes that permit isolation of the labeled proteins. The conjugation reaction is bioorthogonal, i.e., the reaction is rapid and selective for labeled proteins and probes, and effectively inert with respect to other components found in worms. Worm lysates were reacted with sodium dithionite-cleavable Diazo Biotin-DBCO (*i*), and Azf-labeled proteins were isolated by streptavidin affinity chromatography (*ii*). The structure of the biotin reagent has been simplified; the full structure is shown in Fig. 1*B*. (*D*) LC-MS/MS analysis of *myo-2*::Thr412Gly-CePheRS animals fed with triply labeled *E. coli* (heavy arginine, heavy lysine, and Azf) for 24 h. Solid blue diamond markers represent proteins that are expressed exclusively in the pharynx. Open red circle markers represent proteins that are expressed in the pharynx. Open black circle markers represent proteins that have either unknown expression patterns or expression patterns not associated with the pharynx. The proteins discussed in this work are marked. (*E*) Fluorescence microscopy confirmed GFP fluorescence was exclusively localized to pharyngeal muscle cells in *C53C9.2::gfp* (*Left*), *K03E5.2::gfp* (*Center*) and *cpn-4::gfp* (*Right*) animals. (Scale bars: 100 μm.)

in-gel proteolytic digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

We identified and quantified 2,270 proteins across triplicate paired (enriched and unenriched) experiments (Fig. 3*D* and *SI Appendix*, Figs. S12 and 13 and Table S3). Of the quantified proteins, 1,607 (71%) had $(H/L)_E/(H/L)_U$ values greater than one, which indicates that the purification method successfully enriched newly synthesized, cell-specific proteins. Among the enriched proteins were 782 proteins that have expression patterns reported in the literature according to WormBase WS244 (21); of these proteins, 409 are known to be expressed in the pharynx (*SI Appendix*, Table S4). We expected that proteins expressed in pharyngeal muscle cells would be overrepresented among proteins with high $(H/L)_E/(H/L)_U$ values and, indeed, found that of the top 12 proteins quantified, two [TNI-4 (22), a troponin I protein and TNC-2 (23), a troponin C protein] are expressed exclusively in pharyngeal muscle cells. Two proteins [SHL-1 (24), a voltage-gated potassium channel and NCX-2 (25), a sodium-calcium exchanger] are expressed in many muscle cells including pharyngeal muscle cells. A fifth protein F59F4.1 (26) is an acyl-CoA oxidase that is also expressed in the pharynx. Also, three well-known pharyngeal muscle-specific myosin heavy chains were among the top 3% of most highly enriched proteins: MYO-1 (top 1.0%, 22/2,270), MYO-2 (top 1.3%, 29/2,270), and MYO-5 (top 2.6%, 58/2,270). Although the right tail of the $(H/L)_E/(H/L)_U$ distribution contains relatively few members, they represent pharynx-specific proteins: of the 1,100 proteins in our dataset with known expression patterns, 7 of the 18 that are thought to be expressed exclusively in the pharynx have $(H/L)_E/(H/L)_U$ values greater than two ($P = 1.25 \times 10^{-8}$; Fisher's exact test). When we examined a test set of 34 proteins whose genes are highly expressed in body wall muscle, intestinal epithelia, and neuronal cells (27), we found only three in our dataset (*SI Appendix*, Table S5). Two of the three are also expressed in the pharynx (28, 29). Although the absence of a protein from a proteomic dataset cannot be taken as evidence that the protein is absent from the sample, this result is consistent with the hypothesis that the method described here provides an effective means of enriching pharyngeal proteins.

Three of the remaining seven "top-12" proteins in our dataset (C53C9.2, K03E5.2, and CPN-4) share similarity with Calponin-1 (30), a human protein implicated in the regulation of smooth muscle contraction, but their expression patterns have not been reported. To determine whether they are expressed in pharyngeal muscle cells, we generated transgenic *C. elegans* lines that express GFP under control of each of the respective 5′ regulatory regions. We detected strong GFP fluorescence exclusively in pharyngeal muscle cells in *C53C9.2::gfp*, *K03E5.2::gfp* and *cpn-4::gfp* animals (Fig. 3*E*). Calponin-1 has a single calponin homology (CH) and multiple calponin family repeat (CFR) domains. Notably, like its muscle-specific paralog CPN-3, CPN-4 has a CH domain, but no CFRs (*SI Appendix*, Fig. S14). In contrast, C53C9.2 and K03E5.2 have multiple CFRs but no CH domains. Only four *C. elegans* proteins contain multiple CFRs: C53C9.2, K03E5.2, T25F10.6, and UNC-87 (31), a protein required to maintain structure of myofilaments in muscle cells. T25F10.6 (top 5.3%, 121/2,270) and UNC-87 (top 7.5%, 170/2,270) were also among the top 10% of most highly enriched proteins. Although the characterization of new pharyngeal proteins was beyond the scope of this work, their placement among highly enriched proteins, localization, and similarity to other muscle-specific proteins suggest that C53C9.2, K03E5.2, and CPN-4 are excellent candidates for regulating aspects of pharyngeal muscle biology. Together, these results demonstrate that the approach described here can be used to identify proteins (including proteins with previously unknown expression patterns) that are expressed in targeted subsets of cells. We note that in a cell-selective BONCAT experiment, proteins are labeled only after the shift in food source. Although long labeling times can be used to profile the majority of proteins in specific cells, short

labeling times can be used to capture rapid changes in protein expression in those cells.

In summary, by using cell-specific promoters to drive expression of an engineered *Ce*PheRS, we demonstrated that cell-selective BONCAT coupled with SILAC can be used to identify proteins with spatiotemporal selectivity in living *C. elegans*. For future experiments, we suggest several avenues for improvement. First, we incubated worm lysates with Diazo Biotin-DBCO and isolated Azf-labeled proteins by streptavidin affinity chromatography. Because streptavidin affinity chromatography requires mild washing conditions to preserve streptavidin's bioactivity, enrichment quality might be affected by background proteins due to insufficient washing. Alternatively, Azf-labeled proteins can be selectively captured on commercially available alkynyl- or cyclooctynyl-functionalized resins that allow for highly stringent washing conditions to remove nonspecifically bound proteins. Second, we processed samples by SDS/PAGE and in-gel proteolytic digestion before LC-MS/MS analysis, but this approach can introduce contaminants and is time-consuming and laborious. Because chemical tagging of Azf-labeled proteins occurs immediately after animal lysis, our strategy is compatible with new advances in analytical proteomic workflows such as in StageTip-based filter-aided sample preparation (FASP) (32). Third, in the LC-MS/MS analysis, we normalized H/L ratios of enriched proteins to total unenriched proteins derived from the same worm sample. Because the H/L ratio variability depends on different intrinsic rates of protein synthesis in different cells, we advise investigators to additionally normalize H/L ratios of enriched proteins from one cell type to total proteins derived from mixed-stage worms or enriched proteins from another cell type for a more comprehensive analysis of cell-specific proteins.

Finally, our methodology should prove useful in multiple contexts. For example, one could easily build cell-specific proteomic atlases because (*i*) a catalog of cell-specific transcriptional enhancers is readily available and (*ii*) the creation of transgenic organisms is both rapid and routine. In addition, using regulatory elements to drive intersectional patterns of expression, one could restrict labeling to cells that express both elements and, thus, enhance spatiotemporal selectivity with either a FLP recombinase-based (33) or protein reconstitution-based (34) approach. Furthermore, this technique could be used to study protein–protein interactions in a cell-specific manner because aryl azides like Azf are activated upon UV light irradiation to form covalent adducts with proteins in close proximity (35). In principle, the methodology described here could be applied to other organisms in which efficient delivery of noncanonical amino acids is feasible, alleviating the need for cell sorting or laser capture techniques to isolate protein from specific cells in intact organisms.

Materials and Methods

Full details regarding experimental procedures can be found in *SI Appendix, SI Materials and Methods*.

Labeling in *C. elegans*. *C. elegans* strains previously maintained in S medium supplemented with 25 mg/mL *E. coli* OP50 at 20 °C with agitation were pelleted by centrifugation at 1,000 × *g* for 5 min at room temperature, washed three times with S medium and resuspended in S medium supplemented with 25 mg/mL *E. coli* KY14[pKPY514] or KY33[pKPY514]. Following labeling, worms were harvested by centrifugation at 1,000 × *g* for 5 min at room temperature and cleaned by sucrose flotation. Bacterial material was cleared by washing worms with S Medium over a period of 30 min. Worms were pelleted by centrifugation at 1,000 × *g* for 5 min at room temperature and frozen in liquid nitrogen.

Labeling in *E. coli*. To prepare KY14[pKPY514] or KY33[pKPY514], overnight cultures were diluted into freshly prepared M9 medium and agitated at 37 °C until reaching an OD_{600} of 0.5. Cells were pelleted by centrifugation at 5,000 × *g* for 15 min at 4 °C, washed three times with ice-cold 0.9% (wt/vol) sodium chloride, and resuspended in freshly prepared M9 minimal medium (without Phe) supplemented with 2.0 mM Azf. After another 30 min of agitation at 37 °C, expression of Thr251Gly-*Ec*PheRS was induced by the addition of 1 M IPTG. After 4 h of agitation at 37 °C, cells were harvested by centrifugation at 5,000 × *g* for 15 min at 4 °C and resuspended in freshly prepared M9 minimal medium (without Phe) supplemented with 2.0 mM Azf at a concentration of 250 mg of wet cell mass/mL medium and stored at 4 °C.

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BIOCHEMISTRY

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